facilities and Mr. Thomas J. Reed of Chincoteague, Va., for advice and assistance in securing the ponies for the experiments. The authors further wish to thank Drs. C. May, C. Sevy and R. Zinn and the staff of the Animal Hospital Section, Laboratory Aids Branch, National Institutes of Health, for assistance in these studies.

1. Sovinova, B., Tumova, B., Pouska, J., Nemec, J., Col. Epidem., 1957, v6, 213.
2. Waddell, G. H., Teigland, M. B., Sigel, M. M., J.A.M.A., 1963, v143, 586.
3. Doll, E. R., Am. Rev. Resp. Dis., 1961, v85, 48.
4. Lief, F. S., Cohen, D., Am. J. Epid., 1966, v82, 225.
5. Alford, R. H., Kasel, J. A., Lehrich, J. R., Knight, V., unpublished data.
6. Knight, V., in Progress in Medical Virology, Karger, Basel/New York, 1964, 6: 1-26.
7. Shelokov, A., Vogel, J. E., Chi, L., Proc. Soc. Exp. Biol. and Med., 1958, v97, 802.
8. Kärber, G., Arch. P. Exp. Path. u. Pharmakol., 1931, v162, 480.
9. Francis, T., Jr., in Doerr, R., Holloner, C., eds., Handbook der Virusforschung, Springer, Vienna, vol. 3, p66.
10. Knight, V., Kasel, J. A., Alford, R. H., Loda, F., Morris, J. A., Davenport, F. A., Robinson, R. Q., Buescher, E. L., Ann. Int. Med., 1965, v62, 1307.
11. Scholtens, R. G., Steele, J. H., Publ. Health Rep., 1964, v79, 393.
12. Dowdle, W. R., Yarbrough, W. B., Robinson, R. Q., ibid., 1964, v79, 398.
13. Minuse, E., McQueen, J. L., Davenport, F. M., Francis, T., Jr., J. Immunol., 1965, v94, 563.

Received November 4, 1966. P.S.E.B.M., 1967, v124.

Specific Heterophile Antibody from Infectious Mononucleosis Serum.* (31778)

KOSAKU YOSHIDA AND ANDRÉ NAHMIAS (Introduced by Morris Tager)
Department of Pediatrics, Emory University School of Medicine, Atlanta, Ga.

The preparation of a specific form of heterophile antibody (HA) would enable better approaches to characterization of this antibody and its site of formation and function. Although this antibody has been found to be present in the macroglobulin fraction (IgM) of infectious mononucleosis serum (1-3), recent evidence indicates that HA constitutes only a small percentage of the increased macroglobulins found in the serum of patients with this condition(4). Standard methods of preparation of macroglobulins, such as chromatography and electrophoresis, would therefore yield macromolecules other than HA.

In order to prepare a specific antibody, advantage was taken of the specific ability of HA to adsorb to beef red cells. This report presents the requirements for obtaining maximal elution of the adsorbed HA, as well as certain immunological and stability characteristics of the purified antibody.

* Supported by Grant AI-06157 from Nat. Inst. Health, USPHS.

Materials and methods. Antigens. Beef cell antigen (Balt. Biol. Lab.), freshly obtained beef red cell stroma and beef cell antigen (Hyland Lab., Calif.) were used in preliminary experiments. Beef cell antigen (BBL) gave superior results and was used thereafter.

Test sera. Sera were separated from the blood of patients with infectious mononucleosis and were inactivated at 56°C for 30 minutes prior to testing. Sheep hemagglutination activity and adsorption tests with beef red cell and guinea pig kidney antigens (BBL) were done according to standard procedure(5).

Relationship between amounts of beef cell antigen and the adsorbed and eluted HA. To 5 ml of a 1:2 dilution of serum, previously adsorbed with guinea pig kidney, and to each 2-fold dilution of this serum in pH 7.0 M/15 phosphate buffered saline (PBS) were added varying amounts of beef cell antigen (20 mg to 640 mg). The mixture was incubated for 18 hours at 4°C in a shaker. Each sample was then centri-

TABLE I. Relation Between Adsorption and Elution of Heterophile Antibody by Varying Amounts of Beef Cell Antigen.

Antibody titer	Antigen (mg) 20		40		80		160		320		640	
	% A*	% E†	% A	% E	% A	% E	% A	% E	% A	% E	% A	% E
1:10240	20.65	.50	60.00	2.00	97.5	4.00	99.5	4.00	99.9	1.00	100	.10
1:5120	80.00	.50	90.00	2.00	98.7	4.00	99.9	1.00	99.9	0.21	100	0
1:2560	90.39	.62	99.67	2.00	99.06	0.12	100	0.06	100	0	100	0
1:1280	95.00	0	99.90	0.50	100	0	100	0	100	0	100	0
1:640	99.97	0	100	0	100	0	100	0	100	0	100	0
1:320	100	0	100	0	100	0	100	0	100	0	100	0

* A = adsorbed. † E = eluted.

fuged at 3000 *g* for 20 minutes. The supernate was removed and tested for its hemagglutinating activity and the percent adsorbed HA was calculated on the basis of the total antibody titer. The sediment was washed 5 times with a constant amount of buffered saline. Two ml of saline (pH 5) were added to the final sediment and stirred, after which 8 ml of diethyl ether were added and the mixture shaken, then centrifuged at 3000 *g* for 20 minutes. The eluate was present below the top layers of ether and stromal antigen. The top layers were discarded and the eluate layer below kept in a 37°C water bath for 10 minutes to evaporate the ether and centrifuged again to remove particulate matter. The eluate was then tested for sheep hemagglutinating activity and the percent eluted antibody was calculated on the basis of the original antibody titer.

Isolation of HA by elution and further purification procedures. Using a safety ratio (see *Results*) for the amount of beef cell antigen to use with a serum having a particular sheep hemagglutinating titer, elution was carried out as noted above. In addition, repeated elution was done on the sedimented antigen in the bottom layer of the first elution procedure. The non-adsorbed antibody in the supernate was also submitted to the complete elution procedure.

Further purification was performed by sucrose density gradient ultracentrifugation (6). The early fractions which had sheep hemagglutinating activity were collected and concentrated by ultrafiltration.

Immunological tests. The purified concentrated eluates were tested using the Ouchterlony immunodiffusion technique(7) and immunoelectrophoresis(8) against antihuman, anti IgM, anti IgA and anti IgG goat serum (Hyland Lab.).

Stability of eluted HA with various agents and at varying temperatures. The sheep hemagglutinating titer of eluates in PBS was tested at 4°C, —20°C and —70°C. Equal volumes of the eluates in PBS were mixed with the agents listed below and tested after 1 day, 2 days and 2 weeks of storage at 4°C: tyrode solution, gelatin (0.2%), ionagar (0.2%), glycerin (100%), tris NaCl buffer (pH 8.75), sucrose (60%) and PBS.

Results. Relation between adsorption and elution of HA by varying amounts of beef cell antigen. Table I presents results of a representative experiment on the adsorption and elution of HA by varying amounts of beef cell antigen. It can be noted there is an almost direct relationship between amount of antigen and antibody titer for maximal adsorption to occur (diagonal line in Table I). Elution of HA was poor with maximal adsorption. Maximal elution was obtained with a specific ratio of antigen to antibody *e.g.*, when the antibody titer was 1:10,240 maximal elution occurred with 80 to 160 mg of antigens and was less with either smaller or larger amounts of antigen.

A safety ratio which would permit near maximal elution of HA was determined from such experiments to be 1 mg of beef cell antigen to 1:2500 HA titer per 0.25 ml of serum. Elution of HA from sera with

TABLE II. Stability of HA Activity* in Various Diluents at 4°C.

Diluent	Days after incubation 1	2	4	14
Tyrode solution	1:128	1:128	1:128	1:32
Gelatin solution	1:256	1:256	1:256	1:128
Ionagar	1:256	1:256	1:256	1:128
Sucrose	1:256	1:256	1:256	1:256
Tris HCl buff. saline	1:128	1:128	1:128	1:128
Glycerin	1:256	1:256	1:256	1:256
PBS	1:128	1:128	1:128	1:64

* Original HA activity was 1:512 prior to 2-fold dilution with diluent.

varying HA titers obtained from several patients with infectious mononucleosis was performed using the following formula:

$$\text{Antigen (mg)} = \frac{\text{hemagglutination titer} \times \text{vol. of sera (ml)}}{0.25} \times \frac{1}{2500}$$

Elution could be repeated on the sedimented antigen in the bottom layer of the first elution procedure. In addition, further elution of the non-adsorbed supernate antibody was also done. Using such methods, the percent eluted antibody from several patients' sera was found to be between 4 and 10% of the original antibody.

Stability of sheep hemagglutinating activity. It was noted that the sheep hemagglutinating activity of different eluates in M/15 phosphate buffer (7.0) dropped significantly (4-16-fold) after storage at —20°C or 4°C. Stability was determined by using an eluate with an initial titer of 1:5120 which was diluted ten-fold. One ml of the diluted eluate was added to 1 ml of the various media listed in Table II and stored at 4°C for 1, 2, 4 and 14 days. At each of these time intervals the sheep agglutinating activity was ascertained. There was a slight but insignificant drop in sheep agglutinating activity within the first 4 days. However, after 2 weeks the fall in titer was least marked with glycerin and 30% sucrose. Further experiments confirmed that the stability of the antibody was greatest in glycerin or sucrose.

Immunological characteristics. It was found that a 1:2000 sheep hemagglutination titer had to be present in the eluate to obtain a visible precipitin line by immunodiffusion or immunoelectrophoresis when tested with antihuman or anti IgM sera. In eluates obtained from a few infectious mononucleosis sera, a faint line was also observed with anti IgG serum and 2 lines with antihuman sera. Therefore, eluates were further purified by sucrose density gradient ultracentrifugation. Such purified materials reacted only with anti IgM sera and only a line of identity was obtained with antihuman serum (Fig. 1). The sheep hemagglutinating activity of the final product was not adsorbed by guinea pig kidney antigen.

Discussion. Various methods of eluting antibodies from erythrocytes have been previously described—hypertonic solutions(9), heat(10), lowering the pH(11). When these methods yielded poor recovery of heterophile antibody activity from sera of patients with infectious mononucleosis in preliminary experiments, the technic described by Rubin (12) using diethyl ether was employed for these studies.

It was found that the amount of beef cell antigen required for maximal elution of heterophile activity varied with the initial antibody titer of the patient's serum. With excess amounts of antigen, very small, if any, amounts of antibody could be dissociated. Maximal elution was obtained at particular antigen antibody concentrations and a formula was devised for this purpose. The in-

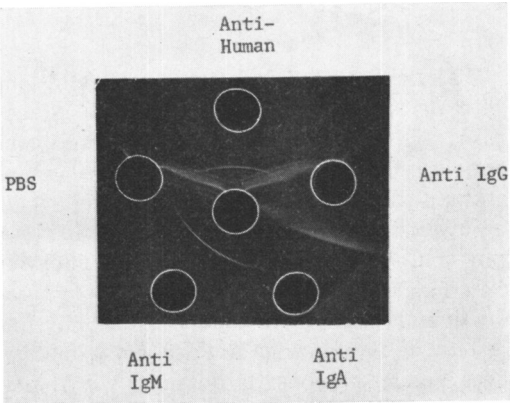

FIG. 1. Ouchterlony double diffusion test. In center well is purified heterophile antibody. Single lines between eluate and antihuman or anti IgM sera are noted. Other precipitin lines in Figure are between antisera themselves.

ability to elute all of the antibodies from the beef cell antigen is probably related to a difference in the avidity of the heterophile antibody molecules(13).

It was also observed that on occasion, small amounts of IgG were adsorbed to the beef cell antigen, necessitating further purification by sucrose density gradient ultracentrifugation. The purified material had the immunological characteristics of heterophile antibody reported by other workers(1-3). However, the lability of this material has proven to be a problem, as noted by others for certain 19S immunoglobulins(14). The stability of our preparation was found to be greatest in 50% glycerin or 30% sucrose at 4°C.

Summary. Specific heterophile antibody was prepared by adsorption to beef erythrocytes and elution with diethyl ether. Using a formula based on the antigen-antibody relationship permitting maximum elution, up to 10% of the antibody was recoverable. The eluted material, further purified by sucrose density gradient ultracentrifugation, had the characteristics of heterophile antibody. The material was found to be unstable at −20°C or 4°C storage, but was relatively stable in 30% sucrose and 50% glycerin at 4°C.

1. Kunkel, H. G., Fudenberg, H. H., Ovary, Z., Ann. N. Y. Acad. Sci., 1960, v86, 966.
2. Grubb, R., Swahn, B., Acta path. et microbiol. Scand., 1958, v43, 305.
3. Strannegard, O., Hanson, L. A., ibid., 1964, v61, 551.
4. Wollheim, F. A., Williams, R. C., New Eng. J. Med., 1966, v274, 61.
5. Davidsohn, I., Lee, C. L., Am. J. Clin. Path., 1964, v41, 115.
6. Kunkel, H. G., The plasma proteins, Academic Press, 1960, 279.
7. Ouchterlony, O., Progr. Allerg., 1958, v5, 1.
8. Scheidigger, J. J., Int. Arch. Allerg., 1955, v7, 103.
9. Komninos, Z. D., Rosenthal, M. C., J. Lab. Clin. Med., 1953, v41, 887.
10. Landsteiner, K., Miller, C. P., J. Exp. Med., 1925, v42, 853.
11. Kidd, P., J. Clin. Path., 1949, v2, 103.
12. Rubin, H., ibid., 1963, v16, 70.
13. Farr, R. S., J. Infect. Dis., 1958, v103, 239.
14. Bauer, D., Stavitsky, A. B., Proc. Nat. Acad. Sci., 1961, v47, 166.

Received November 10, 1966. P.S.E.B.M., 1967, v124.

Influence of Prior Sensitization with Mycobacteria on Antibody Response To Protein Antigen in Freund's Adjuvants. (31779)

STEPHEN A. ARON AND BERNARD W. JANICKI
Basic Microbiology Research Laboratory, Veterans Administration Hospital, Washington, D. C.

The wide use of water-in-oil emulsions with or without killed mycobacteria to enhance the immune response reflects the general acceptance of the Freund-type preparations as effective and potent immunologic adjuvants. In general, injection of an antigen in a water-in-oil emulsion (Freund's incomplete adjuvant) results in antibody production greater than that obtained by injection of the aqueous antigen alone and an even greater response is obtained when various mycobacteria are added to the emulsion (Freund's complete adjuvant). However, the antibody enhancing effect of such adjuvants may be altered by the immunization regimen. Weigle and his associates(1) found that the effect of the complete adjuvant on production of precipitating antibody to a constant amount of protein antigen in guinea pigs was no greater than that of the incomplete when the preparations were administered in multiple, large volume, doses. In contrast, the complete adjuvant had an extreme enhancing effect when an equal amount of antigen was given in a single, small volume, injection. Because the mycobacteria themselves function as an antigenic stimulus and sensitize the host to tuberculin, the possibility exists that prior sensitization with mycobacteria may also influence the antibody en-